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Two Transcriptionally Active OmpR Mutants That Do Not Require Phosphorylation by EnvZ in an *Escherichia coli* Cell-Free System

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D55Q-T83A and D55Q-G94S, two pseudorevertants of the D55Q mutant OmpR, an *Escherichia coli* transcriptional activator, were isolated previously by R. Brissette, K. Tsung, and M. Inouye (J. Bacteriol. 173:3749–3755, 1991). These pseudorevertant OmpR proteins were purified and examined for their function as transcriptional activators in a cell-free system with an *ompF* DNA fragment. These proteins were transcriptionally active, even after acid treatment, whereas the wild-type OmpR was completely inactive after the same treatment. Phosphorylation of acid-treated wild-type OmpR with an EnvZ11 membrane fraction and ATP restored transcriptional activity, whereas the activities of the mutant OmpR proteins did not change after phosphorylation.

One way *Escherichia coli* responds to osmotic changes in the environment is by altering the levels of OmpF and OmpC, two outer membrane porin proteins. Under low osmotic conditions OmpF is preferentially produced, whereas under high osmotic conditions OmpF production is repressed and OmpC production is induced (11, 18, 24). This process is regulated by two proteins encoded by the *ompB* locus: EnvZ, an inner transmembrane protein (9, 12), and OmpR, a cytoplasmic transcriptional activator protein for *ompF* and *ompC* (1, 13, 16). EnvZ and OmpR belong to a family of sensory-regulatory protein pairs that control such diverse processes as nitrogen fixation, chemotaxis, and phosphate regulation (for a review, see reference 23). The EnvZ-like sensory proteins have sequence homologies at their C-terminal domains, and the OmpR-like regulatory proteins have homologies at their N-terminal domains. In a manner similar to that of other protein pairs in the family, EnvZ has been shown to serve as both a kinase and a phosphatase for OmpR (2–4, 10, 13, 14).

EnvZ undergoes autophosphorylation, most likely at a conserved histidine residue in the C-terminal domain (10, 15), and then the phosphate group is transferred to OmpR. The site of OmpR phosphorylation is believed to be D55, one of the three conserved aspartic acid residues in the N-terminal domain of OmpR (22). Phosphorylated OmpR promotes transcription from the *ompF* and *ompC* promoters (1, 13). This increased level of expression is most likely due to an increased ability of OmpR phosphate to bind both promoter regions (4). In the current model of osmoregulation, EnvZ, the osmosensor in the inner membrane, senses changes in osmolarity and relays this signal to OmpR through phosphorylation. OmpR phosphate then binds to the specific sites upstream of the *ompF* and *ompC* promoters to regulate expression of the genes accordingly.

In a previous study by Brissette et al. (6), two pseudorevertants of the D55Q mutation were isolated. The phosphorylation mutant, D55Q OmpR, gave an $OmpF^-$ $OmpC^-$ phenotype. However, the pseudorevertant OmpRs, D55Q-T83A and D55Q-G94S, reverted the $OmpF^-$ $OmpC^-$ phenotype back to $OmpF^+$ $OmpC^+$ in *ompB* deletion ($OmpR^-$) cells. Expression of OmpF in cells producing the revertant OmpRs correlated with a restored binding of the revertant OmpR to the promoter region. Both an in vivo transcription assay and an in vitro phosphorylation experiment indicated that the pseudorevertants functioned in an EnvZ-independent manner. However, because of cross-talk mechanisms by other EnvZ-like histidine kinases in *E. coli* (23), the phosphorylation independence of these mutants had to be further confirmed in vitro. In this study we used in vitro assays to demonstrate that the nonphosphorylated forms of the two OmpR mutants (D55Q-T83A and D55Q-G94S) are indeed transcriptionally active, whereas the wild-type OmpR is only active in the phosphorylated form.

In vitro transcription of *ompF*. A 330-bp *Xba*I-*Bam*HI fragment from plasmid pKI0033 (21) was used in an in vitro assay system modified by us from that described by Okamoto and Freundlich (22). This fragment encompasses the *ompF* promoter region from −173 to +122 and thus contains the OmpR binding motifs (Fa, Fb, Fc, Cd) as well as the −35 and −10 regions. OmpR purified by the procedure of Norioka et al. (21) was incubated with the *ompF* promoter fragment (0.2 pmol) in a total volume of 19.5 μl for 15 min at room temperature. RNA polymerase (0.1 U per reaction; U.S. Biochemical Corp.) was added, and the mixture was further preincubated at 37°C for 5 min. The ribonucleotide mixture (2.7 mM with respect to ATP, GTP, and CTP, 0.0625 mM with respect to UTP, and 0.8 μCi of [α-^{32}P]UTP [3,000 Ci/mmol; Amersham]), 8 mM magnesium acetate, and 0.1 mg of rifampin per ml were added, and incubation was continued for 10 min. The reaction was stopped by the addition of 10 μl of formamide dye solution (98% deionized formamide, 2 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue), incubated at 90°C for 3 min, and then loaded onto an 8% polyacrylamide sequencing gel. The wild-type, D55Q-T83A, and D55Q-G94S OmpRs were transcriptionally active (Fig. 1, lanes 2, 3, and 4, respectively). It should be noted that the wild-type OmpR that had not been phosphorylated in vitro with EnvZ and ATP before being used in the transcription assay activated transcription, albeit

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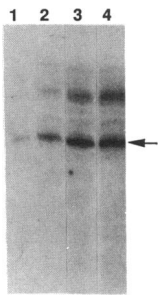

FIG. 1. In vitro transcription of the *ompF* promoter. In vitro transcription of the *ompF* promoter (0.2 pmol) in the presence of purified OmpR (15 ng) was carried out as described in the text. Lanes: 1, no added OmpR; 2, wild-type OmpR; 3, D55Q-T83A; 4, D55Q-G94S. The arrow indicates the position of the *ompF* transcript.

weakly. To determine whether this was due to a residual amount of phosphorylated OmpR in the OmpR preparation, the wild-type OmpR was subjected to chemical dephosphorylation under acid conditions (pH 1 at 37°C for 50 min). The two mutant OmpRs were also subjected to the same treatment. The conditions employed for the chemical dephosphorylation had been shown previously by Forst et al. (10) to remove 95% or more of acyl-phosphate from OmpR.

Phosphorylation independence of mutant OmpRs. After treatment with mild acid followed by neutralization, the three OmpRs were again assayed for transcriptional activity. This time (Fig. 2, lanes 2 through 4), the wild-type OmpR was completely inactive even at high concentrations (90 ng per reaction). In contrast, acid treatment had no effect on the mutant OmpRs; they still activated *ompF* transcription. It should be noted that as the concentrations of OmpR increased the production of the *ompF* transcript started to be inhibited. It was shown previously by Aiba et al. (2) that *ompF* transcription can be repressed by increasing the amounts of phosphorylated OmpR in vitro. This inhibitory effect on *ompF* transcription by a higher concentration of OmpR probably reflects the repressor function of phosphorylated OmpR at high osmolarity in vivo (12).

EnvZ dependence of wild-type OmpR. To confirm that the

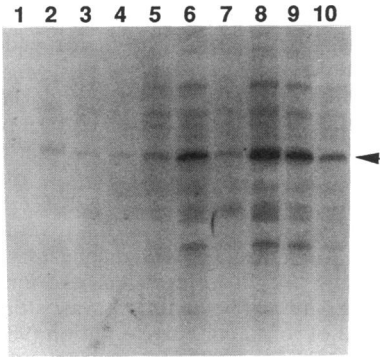

FIG. 2. Transcriptional activity of dephosphorylated OmpR. Purified OmpRs were chemically dephosphorylated with mild acid and neutralized as described in the text. Lanes: 1, no added OmpR; 2 through 4, acid-treated wild-type OmpR (10, 30, and 90 ng, respectively); 5 through 7, acid-treated D55Q-T83A (10, 30, and 90 ng, respectively); 8 through 10, acid-treated D55Q-G94S (10, 30, and 90 ng, respectively). The arrowhead indicates the position of the *ompF* transcript.

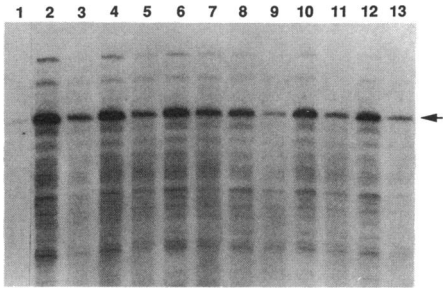

FIG. 3. Phosphorylation dependence of wild-type OmpR. Acid-treated and non-acid-treated OmpRs were phosphorylated in vitro with an EnvZ11 membrane fraction and ATP. Lanes: 1, no OmpR added; 2 through 7, non-acid-treated OmpR; 2 and 3, wild-type OmpR (7.5 and 22.5 ng, respectively); 4 and 5, D55Q-T83A (7.5 and 22.5 ng, respectively); 6 and 7, D55Q-G94S (7.5 and 22.5 ng, respectively); 8 through 13, acid-treated OmpRs; 8 and 9, wild-type OmpR (7.5 and 22.5 ng, respectively); 10 and 11, D55Q-T83A (7.5 and 22.5 ng, respectively); 12 and 13, D55Q-G94S (7.5 and 22.5 ng, respectively). The arrow indicates the position of the *ompF* transcript.

inability of the wild-type OmpR treated with acid to transcribe the *ompF* promoter was not due to the denaturation of the protein by the acid treatment, the wild-type OmpR was phosphorylated with a membrane fraction containing EnvZ11. EnvZ11 is known to have a very high OmpR kinase activity but no phosphatase activity (19). The membrane fraction was prepared from *E. coli* by the method of Yang and Inouye (25). Five microliters of the membrane fraction (4 mg/ml) was first preincubated in 30 μl with 2 mM ATP for 10 min at room temperature. OmpR (1 μl; 500 ng/μl) was then added, and incubation was continued for another 5 min. Phosphorylated OmpR was then separated from the EnvZ11 membrane fraction by centrifugation. The mutant OmpRs were treated in the same manner. These EnvZ11-treated OmpRs were then used in the in vitro transcription assay. The acid-treated wild-type OmpR recovered its transcriptional activator function as a result of its treatment with EnvZ11 and ATP (compare lane 8 in Fig. 3 with lanes 2 through 4 in Fig. 2). The activity was a little lower than that of non-acid-treated OmpR that was treated with EnvZ11 and ATP (Fig. 3, lane 2), probably because some portion of OmpR was indeed denatured by the acid treatment. Similarly, acid-treated mutant D55Q-T83A (lanes 4 and 10) and D55Q-G94S (lanes 6 and 12) OmpRs were both transcriptionally active. Again at higher concentrations, all OmpRs were inhibitory (lanes 3, 5, 7, 9, 11, and 13). These results clearly demonstrate that the wild-type OmpR requires phosphorylation by EnvZ, whereas the pseudorevertant OmpRs do not.

In the present study we further confirm the previously suggested (6) phosphorylation independence of the revertant D55Q-T83A and D55Q-G94S OmpRs. We found that treatment of the wild-type OmpR to remove acyl-phosphate residues resulted in a significant loss of OmpR function. However, the activities of the D55Q-T83A and D55Q-G94S OmpRs were not affected like that of wild-type OmpR was; the revertant OmpRs remained transcriptionally active before and after the treatment. It is assumed (22) that phosphorylation of OmpR causes a conformational change that promotes binding of the C terminus to the specific OmpR-binding sites upstream of the *ompF* and *ompC* promoters and thus activates transcription. Because of the nonconsensus nature of the −10 and −35 promoter regions, transcrip-

tion of *ompF* and *ompC* in the absence of OmpR is negligible. The combination of D55Q with either T83A or G94S, which restores OmpR function, seems to generate a conformational change in the OmpR molecule similar to that caused by phosphorylation, most likely at the aspartic acid residue at position 55. Phosphorylation-independent mutants of CheY (5), a response regulator in the chemotaxis system, and NtrC (8), a response regulator in the nitrogen regulation system, have also been isolated. The nonphosphorylated CheY mutant CheY13DK resulted in a constitutive tumbly phenotype, but, unlike NtrC and OmpR, CheY has no C-terminal DNA binding domain. NtrC is an OmpR-like transcriptional activator in the nitrogen regulation system, but unlike the situation in OmpR, the mutations that give rise to the phosphorylation-independent mutations of NtrC are in the C-terminal DNA binding domain, not the N-terminal activation domain. These mutants, although phenotypically phosphorylation independent, are mechanistically different from the OmpR mutants D55Q-T83A and D55Q-G94S. Kanamuru and Mizuno (17), however, have isolated an EnvZ-independent mutant OmpR that is similar to the two isolated by Brissette et al. The mutation OmpR55Q/102C was isolated as a suppressor to the inactive D55Q OmpR. These three suppressor mutations all occur within a central highly conserved region of the protein. Brissette et al. (7) and Nakashima et al. (20) have isolated OmpR mutants defective in DNA binding but capable of being phosphorylated with mutations in this same highly conserved region. These results suggest that this region may be important for the adoption of a transcriptionally active conformation of OmpR.

The mechanism by which phosphorylation regulates OmpR function at present still remains unclear. Further in vitro characterization of these phosphorylation-independent OmpRs will help to answer this question, which is an important aspect of the role played by OmpR in osmoregulation.

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